

AWARD NUMBER: W81XWH-10-1-0226

TITLE: Function of ZFAND3 in the DNA Damage Response

PRINCIPAL INVESTIGATOR: Bianca M. Sirbu

CONTRACTING ORGANIZATION: Vanderbilt University
Nashville, TN 37232

REPORT DATE: June 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 June 2011		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 Jun 2010 – 31 May 2011	
4. TITLE AND SUBTITLE Function of ZFAND3 in the DNA Damage Response				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-10-1-0226	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bianca M. Sirbu E-Mail: bianca.m.sirbu@vanderbilt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Nashville, TN 37232				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The DNA-damage response (DDR) functions to maintain genomic integrity and prevent tumorigenesis. Cells in human precancerous lesions contain high levels of DNA damage, potentially resulting from unresolved problems accrued during DNA replication. Such replication stress can arise from stalling of replication forks at DNA lesions. The dynamic coordination of mechanisms that detect and repair these damaged replication forks is the subject of this research. Several mechanisms to repair a damaged fork and restart replication depend on tumor-suppressor proteins such as BRCA1 (breast cancer 1) and checkpoint kinase signaling via ATR and ATM. A significant unanswered question is how these DDR proteins are spatially and temporally regulated during replication stress. We have developed a new method termed iPOND (isolation of proteins on nascent DNA) to examine DDR protein dynamics and functions at moving and stalled replication forks in mammalian cells. We used iPOND to define the dynamics of chromatin assembly and maturation. Furthermore, we show that stalled and collapsed replication forks contain different DDR protein complexes. Persistent replication fork stalling leads to fork collapse and recruitment of DSB repair proteins such as Rad51. Our systematic study of these mechanisms reveals new insights into how damaged forks are repaired and why defects in specific DDR pathways in cancer can cause genome instability during DNA replication. These studies have implications for understanding breast cancer etiology.					
15. SUBJECT TERMS genome maintenance, replication, DNA damage response, epigenome maintenance					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
BODY.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	9
References.....	10
Appendices.....	13

Introduction

Maintaining genome integrity is essential to prevent carcinogenesis (1,2). During DNA replication, genome maintenance requires that several dynamic processes be coordinated at the replication fork (3,4). Damage encountered during DNA replication, termed replication stress, stalls the replication fork and challenges the accurate completion of genome and epigenome inheritance. The DNA damage response (DDR) becomes activated in response to replication stress to protect, repair, and promote successful completion of chromosome replication (3,5). Importantly, pre-cancerous lesions, such as those of the breast, exhibit high levels of replication stress that activate the DDR (6,7). However, the underlying mechanisms that result in genome instability and uncontrolled cell cycle in these lesions remain unclear and are significant to understanding breast cancer etiology (7).

Upon replication stress, the activated DDR signals to recruit protein complexes to stalled replication forks to maintain genome and epigenome integrity. In addition to localization, DDR proteins can be post-translationally modified at stalled forks to modulate protein dynamics (3). For example, recruitment of the ATR (ATM-and Rad3 related) kinase to stalled replication forks initiates DDR signaling and regulation through a cascade of phosphorylation events (3). One target of ATR after replication stress is the breast cancer susceptibility gene BRCA1 (8).

Our understanding of DDR signaling has benefited from genomic screens that have identified potential regulators of genome integrity, such as ZFAND3 (zinc finger AN1-type domain containing protein 3) (9). Furthermore, studying genome maintenance proteins has relied heavily on detection of protein localization to sites of fork stalling. Unfortunately, in human cells, current methods for studying protein dynamics at replication forks are limited in sensitivity and spatiotemporal resolution (10). To overcome this challenge and analyze protein dynamics at active, stalled and collapsed replication forks, we have developed a new methodology termed iPOND (isolation of proteins on nascent DNA) (11). We used iPOND to define the dynamics of proteins and post-translational modification in the replisome (the proteins necessary to complete DNA replication), particularly after replication stress. Replication stress compromises not only genome, but also epigenome integrity (12). Chromatin alterations have been hypothesized to present a source of genomic instability in cancer (13). Therefore, our iPOND analyses included studies of changes that accompany chromatin deposition and maturation during DNA

replication. Overall, we demonstrate the power of iPOND for studies of replisome and chromatin dynamics during normal and replication stress conditions.

Body

The initial goal of the proposed research project was to elucidate the function of the novel DNA damage response gene ZFAND3. Several lines of evidence placed ZFAND3 in the DDR pathway. ZFAND3 accumulates after DNA damage and functions to sustain the G2/M checkpoint after ionizing radiation (IR). Furthermore, ZFAND3 was identified to interact with the breast cancer gene TOPBP1 (topoisomerase binding protein 1) in a yeast two-hybrid screen. Lastly, ZFAND3 depletion sensitized to PARP inhibition. Synthetic lethality with PARP is significant since therapeutic gain has been reported in patients lacking the breast cancer susceptibility gene BRCA1 (14).

As indicated in the statement of work, the first aim was to characterize the function of ZFAND3 in the DDR. In accordance with this, we found that depletion of ZFAND3 caused minimal reduction in checkpoint signaling through Chk1 phosphorylation after IR, but not UV or HU treatments. To address ZFAND3 knockdown efficiency in the loss of function assays, we raised an antibody to ZFAND3. However, the antibody recognized only exogenously expressed tagged protein, which forced temporary suspension of task 1.

The subsequent task was to identify ZFAND3 interacting proteins. In agreement with the proposed outline, we sought to confirm the yeast two-hybrid interaction between TOPBP1 and ZFAND3 using pulldown methodologies and co-immunoprecipitation (co-IP). Recombinant GST-tagged TOPBP1 fragments and full-length protein were purified and incubated with cell lysates expressing tagged ZFAND3. No interaction between TOPBP1 and ZFAND3 was detectable before or after IR damage. Co-IPs corroborated the pulldown results under all lysis conditions tested. Therefore, the interaction identified in the yeast two-hybrid screen could not be confirmed using the outlined methods. The unavailability of a ZFAND3 antibody made further protein-protein interaction studies difficult.

The final task of the proposal was to examine the mechanism of ZFAND3 accumulation after genotoxic stress. To this end, the stability of HA-tagged protein was determined using pulse-chase

methods with cycloheximide. The ZFAND3-HA half-life did not change following various treatment times and doses of IR. This suggested that accumulation of tagged ZFAND3 was not due to protein stabilization after damage, as is the case for other DDR proteins such as p53 (15). Furthermore, aggregation of ZFAND3-HA was found to be clonal and cell-type dependent. Therefore, the subsequent experiments aimed to understand checkpoint regulation of ZFAND3 levels were suspended.

At this point, we completed the list of tasks in the outlined experimental design that could be performed without the essential tool—a good ZFAND3 antibody. With insufficient tools and evidence to pursue ZFAND3 function as a TOPBP1 interacting protein that accumulates after DNA damage, we redirected our studies to encompass a broader understanding of genome maintenance activities that prevent breast cancer.

To maintain genome integrity, numerous dynamic processes must be coordinated and executed with speed and accuracy at the replication fork (3). Exposure of cells to damaging agents during DNA replication can stall replication forks and recruit DDR protein complexes to maintain genome integrity at stalled forks (3,5). Understanding such dynamic activities requires identification of proteins located at the replication fork. In mammalian cells, it has been difficult to stall replication forks at predictable genomic loci, making chromatin immunoprecipitation (ChIP)-based methods inapplicable for isolation of stalled replisomes. ChIP of specific endonuclease-induced DSBs has greatly facilitated understanding of the DDR after DSBs (16,17,18). To provide a tool to study the DDR at moving and stalled replication forks, we have developed a novel method termed iPOND (isolation of proteins on nascent DNA) (11).

The iPOND methodology identifies proteins at replication forks via a single-step streptavidin purification of biotin-tagged nascent DNA and the associated proteins. We validated that iPOND specifically isolates replisome components, even those that are present at one or two copies per replication fork, such as polymerase epsilon. Therefore, iPOND provides improved sensitivity of detection for replisome components compared to the commonly utilized method of immunofluorescence. Of note, iPOND can purify replication proteins that are associated either directly or indirectly with nascent DNA (11).

In addition to improved sensitivity, combining iPOND with pulse-chase methodologies provides high spatial and temporal examination of the changing replisome (11). For example, the replication

protein PCNA (proliferating cell nuclear antigen) is recycled from completed Okazaki fragments on the lagging strand; however, the timing of this event has remained undetermined in mammalian cells (19). Using iPOND pulse-chase, we defined this timing of PCNA recycling, as well as the timing of chromatin maturation. Furthermore, iPOND can monitor the *in vivo* assembly of chromatin and confirmed proposed models of a step-wise deposition of histones onto nascent DNA (11,20).

To understand the DDR to replication stress, we next analyzed the dynamics of proteins recruited and modified at stalled and collapsed replication forks. We showed that shortly upon exposure to the replication stress agent hydroxyurea (HU), the histone variant H2AX is phosphorylated (termed γ H2AX) at stalled replication forks. Phosphorylated H2AX is typically considered a marker of DSBs (21). However, we detected γ H2AX early in the response to HU, prior to evidence of DSB formation at a stalled fork (22). Early γ H2AX corresponded to phosphorylation of RPA on Ser33, an ATR kinase target site (23). The subsequent RPA phosphorylation on Ser4/8, which are DNA-PK sites, occurred after prolonged exposure to replication stress (23). This suggests that ATR phosphorylates RPA immediately after fork stalling, while DNA-PK phosphorylates RPA at persistently stalled forks.

Prolonged exposure to replication stress exhibited enrichment of DSB repair proteins Mre11, KU70/80 and the recombinase Rad51 at stalled forks. Our data suggest that persistently stalled forks collapse into DSBs within four hours of fork stalling. Furthermore, accumulation of Rad51 to persistently, but not transiently stalled forks depends on the nuclease activity of Mre11, similarly to the mechanism that recruits Rad51 to DSBs (24).

At DSBs, γ H2AX spreads from the DSB to facilitate DDR signaling (25,26). Several observations of γ H2AX patterns at stalled forks led us to test whether γ H2AX also spreads from a stalled replication fork. Indeed, using various iPOND pulse-chase methods, we showed that γ H2AX spreads from stalled replication forks to include a large chromatin domain. Of note, the propagation of γ H2AX occurs shortly after exposure to replication stress. To examine the checkpoint kinases responsible for initiating and spreading γ H2AX, we tested the necessity of ATR, ATM and DNA-PK in time course experiments. Our results are consistent with a model wherein ATR phosphorylates H2AX at stalled forks and promotes initial γ H2AX spreading. Longer exposures to replication stress require the combined activities of ATR, ATM, and DNA-PK to initiate, spread and maintain γ H2AX at the stalled fork and in regions proximal to the fork.

The iPOND methodology and findings described above have been incorporated into a manuscript that has been published in *Genes and Development* (see appendix).

Key Research Accomplishments

- Completion of proposed plan for characterization of ZFAND3 in the DDR
- Development of a novel methodology iPOND (isolation of proteins on nascent DNA) to study the DDR at stalled replication forks in mammalian cells at high spatial and temporal resolution
- Validation of iPOND to show replisome dynamics and the timing of chromatin assembly and maturation
- Characterization of dynamic replisome composition at stalled and collapsed replication forks
- DSB repair proteins are recruited to persistently stalled replication forks
- γ H2AX spreads from stalled replication forks in an ATR, ATM, and DNA-PK dependent manner
- Rad51 protein accumulation at persistently stalled replication forks depends on Mre11 nuclease activity

Reportable outcomes

1. Publication of iPOND as novel method to study genome maintenance activities at replication forks in mammalian cells
 - a. Title: Analysis of protein dynamics at active, stalled and collapsed replication forks
 - b. Authors: **Sirbu BM**, Couch FB, Feigerle JT, Bhaskara S, Hiebert SW, Cortez D
 - c. Published: June 2011 *Genes and Development*. 25(12):1320-7
2. Oral presentation of iPOND methodology
 - a. Title: Analysis of protein dynamics at active, stalled and collapsed replication forks
 - b. Speaker: **Sirbu BM**
 - c. Meeting: Gordon Research Conference, Cell Growth and Proliferation, June 29, 2011, Biddeford, ME.
3. Poster presentation of iPOND methodology
 - a. Title: Analysis of protein dynamics at active, stalled and collapsed replication forks
 - b. Authors: **Sirbu BM**, Couch FB, Feigerle JT, Bhaskara S, Hiebert SW, Cortez D
 - c. Meeting: Gordon Research Seminar, Cell Growth and Proliferation, June 26, 2011, Biddeford, ME.
4. Poster presentation describing the iPOND methodology
 - a. Title: Analysis of protein dynamics at active, stalled and collapsed replication forks
 - b. Authors: **Sirbu BM**, Couch FB, Feigerle JT, Bhaskara S, Hiebert SW, Cortez D
 - c. Retreat: Vanderbilt Ingram Cancer Center Retreat, Nashville, TN, May 2011

Conclusions

Genome integrity must be maintained at the replication fork to prevent cancer (3). Accurate completion of chromosome replication involves the spatial and temporal coordination of genome maintenance protein dynamics at replication forks. Previous studies of the replisome during normal and stress responses at stalled forks have relied largely on immunofluorescent imaging to track protein localization. While useful, immunofluorescence (IF) has the significant disadvantages of low resolution and low sensitivity. For example, proteins that exist at only single copy levels at replication forks cannot be tracked with IF. To overcome this difficulty, we have developed a new methodology termed iPOND to isolate proteins on nascent DNA. iPOND has dramatically improved sensitivity compared to IF and allows the interrogation of dynamic events that occur at the normal and stalled fork with high spatial and temporal resolution.

We used this method to define the timing of histone deposition and chromatin maturation. We found that replication fork stalling causes changes in the recruitment and phosphorylation of proteins at the damaged fork. H2AX phosphorylation, typically considered a DSB marker, occurs at stalled replication forks and spreads to include chromatin domains away from the replication fork. This occurs in a checkpoint dependent manner prior to evidence of DSB formation at a stalled fork. Finally, we demonstrated a switch in the DDR at persistently stalled forks that includes Mre11-dependent Rad51 assembly.

Overall the data we have obtained with iPOND provides the first high-resolution, time-dependent analyses of protein dynamics at active, stalled, and collapsed replication forks in mammalian cells. Furthermore, our results validate iPOND as a powerful method to study DNA damage responses, chromatin deposition, and chromatin maturation during DNA replication. These dynamic processes must be well defined to comprehend how their coordination during replication stress prevents the genomic and epigenomic instability that is a hallmark of cancerous lesions, such as those found in breast cancer.

References

1. Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. *Nature* 411:366-74.
2. Schar, P. (2001). Spontaneous DNA damage, genome instability, and cancer—when DNA replication escapes control. *Cell* 104:329-32.
3. Cimprich, K.A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* 9, 616-627.
4. Probst, A.V., Dunleavy, E., and Almouzni, G. (2009). Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* 10, 192-206.
5. Harper, J.W., and Elledge, S.J. (2007). The DNA damage response: ten years after. *Mol Cell* 28, 739-745.
6. Bartkova J, Horejsí Z, Koed K, Krämer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Ørntoft T, Lukas J, Bartek J. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434:664-70.
7. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Dittullo RA Jr, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD. (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 434:907-13.
8. Tibbetts RS, Cortez D, Brumbaugh KM, Scully R, Livingston D, Elledge SJ, Abraham RT. (2000). Functional interaction between BRCA1 and the checkpoint kinase ATR during genotoxic stress. *Genes Dev* 14(23):2989-3002.
9. Polo S.E., Jackson S.P. (2011). Dynamics of DNA damage response proteins on DNA breaks: a focus on protein modifications. *Genes Dev* 25(5):409-33.
10. Sirbu BM, Couch FB, Feigerle JT, Bhaskara S, Hiebert SW, Cortez D. (2011). Analysis of protein dynamics at active, stalled and collapsed replication forks. *Genes Dev* 25(12):1320-7.
11. Lovejoy CA, Xu X, Bansbach CE, Glick GG, Zhao R, Ye F, Sirbu BM, Titus LC, Shyr Y, Cortez D. (2009). Functional genomic screens identify CIMP as a genome maintenance protein. *Proc Natl Acad Sci U S A* 106(46):19304-9.

12. Jasencakova Z, Scharf AN, Ask K, Corpet A, Imhof A, Almouzni G, **Groth** A. (2010). Replication stress interferes with histone recycling and predeposition marking of new histones. *Mol Cell* 37(5):736-43.
13. Jasencakova Z, **Groth** A. (2010). Replication stress, a source of genetic aberrations in cancer. *Bioessays* 32(10):847-55.
14. Ashworth, A. (2008). A synthetic lethal therapeutic approach: Poly(ADP) Ribose Polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. *J Clin Oncol* 26(22):3785-90.
15. Kubbutat MH, Jones SN, Vousden KH. (1997). Regulation of p53 stability by Mdm2. *Nature* 387(6630):299-303.
16. Rodrigue, A., Lafrance, M., Gauthier, M.C., McDonald, D., Hendzel, M., West, S.C., Jasin, M., and Masson, J.Y. (2006). Interplay between human DNA repair proteins at a unique double-strand break in vivo. *EMBO J* 25, 222-231.
17. Rudin, N., and Haber, J.E. (1988). Efficient repair of HO-induced chromosomal breaks in *Saccharomyces cerevisiae* by recombination between flanking homologous sequences. *Mol Cell Biol* 8, 3918-3928.
18. Soutoglou, E., Dorn, J.F., Sengupta, K., Jasin, M., Nussenzweig, A., Ried, T., Danuser, G., and Misteli, T. (2007). Positional stability of single double-strand breaks in mammalian cells. *Nat Cell Biol* 9, 675-682.
19. Shibahara K, **Stillman** B. (1999). Replication dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* 96(4):575-85.
20. Worcel, A., Han, S., and Wong, M.L. (1978). Assembly of newly replicated chromatin. *Cell* 15, 969-977.
21. Dickey, J.S., Redon, C.E., Nakamura, A.J., Baird, B.J., Sedelnikova, O.A., and Bonner, W.M. (2009). H2AX: functional roles and potential applications. *Chromosoma* 118, 683-692.
22. Petermann, E., and Helleday, T. (2010). Pathways of mammalian replication fork restart. *Nat Rev Mol Cell Biol* 11, 683-687.

23. Anantha, R.W., Vassin, V.M., and Borowiec, J.A. (2007). Sequential and synergistic modification of human RPA stimulates chromosomal DNA repair. *J Biol Chem* 282, 35910-35923.
24. Mimitou, E.P., and Symington, L.S. (2009). DNA end resection: many nucleases make light work. *DNA Repair (Amst)* 8, 983-995.
25. Berkovich, E., Monnat, R.J., Jr., and Kastan, M.B. (2008). Assessment of protein dynamics and DNA repair following generation of DNA double-strand breaks at defined genomic sites. *Nat Protoc* 3, 915-922.
26. Savic, V., Yin, B., Maas, N.L., Bredemeyer, A.L., Carpenter, A.C., Helmink, B.A., Yang-lott, K.S., Sleckman, B.P., and Bassing, C.H. (2009). Formation of dynamic gamma-H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent upon H2AX densities in chromatin. *Mol Cell* 34, 298-310.



Analysis of protein dynamics at active, stalled, and collapsed replication forks

Bianca M. Sirbu, Frank B. Couch, Jordan T. Feigerle, et al.

Genes Dev. 2011 25: 1320-1327

Access the most recent version at doi:[10.1101/gad.2053211](https://doi.org/10.1101/gad.2053211)

Supplemental Material <http://genesdev.cshlp.org/content/suppl/2011/06/17/25.12.1320.DC1.html>

References This article cites 43 articles, 16 of which can be accessed free at:
<http://genesdev.cshlp.org/content/25/12/1320.full.html#ref-list-1>

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#)

To subscribe to *Genes & Development* go to:
<http://genesdev.cshlp.org/subscriptions>

Analysis of protein dynamics at active, stalled, and collapsed replication forks

Bianca M. Sirbu, Frank B. Couch, Jordan T. Feigerle, Srividya Bhaskara, Scott W. Hiebert, and David Cortez¹

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA

Successful DNA replication and packaging of newly synthesized DNA into chromatin are essential to maintain genome integrity. Defects in the DNA template challenge genetic and epigenetic inheritance. Unfortunately, tracking DNA damage responses (DDRs), histone deposition, and chromatin maturation at replication forks is difficult in mammalian cells. Here we describe a technology called iPOND (isolation of proteins on nascent DNA) to analyze proteins at active and damaged replication forks at high resolution. Using this methodology, we define the timing of histone deposition and chromatin maturation. Class 1 histone deacetylases are enriched at replisomes and remove predeposition marks on histone H4. Chromatin maturation continues even when decoupled from replisome movement. Furthermore, fork stalling causes changes in the recruitment and phosphorylation of proteins at the damaged fork. Checkpoint kinases catalyze H2AX phosphorylation, which spreads from the stalled fork to include a large chromatin domain even prior to fork collapse and double-strand break formation. Finally, we demonstrate a switch in the DDR at persistently stalled forks that includes MRE11-dependent RAD51 assembly. These data reveal a dynamic recruitment of proteins and post-translational modifications at damaged forks and surrounding chromatin. Furthermore, our studies establish iPOND as a useful methodology to study DNA replication and chromatin maturation.

[**Keywords:** DNA replication; chromatin; DNA damage response; H2AX; histone acetylation; EdU; click chemistry]

Supplemental material is available for this article.

Received March 22, 2011; revised version accepted May 16, 2011.

In human cells, more than 6 billion base pairs of DNA need to be replicated and packaged into chromatin every cell division cycle. Failures lead to mutation, epigenetic changes, and other chromosomal aberrations that ultimately cause diseases such as cancer. DNA replication is coordinated with chromatin assembly (Probst et al. 2009). The replisome, containing the proteins necessary to complete replication, is a dynamic machine that must work with speed and precision. DNA lesions, insufficient nucleotides, and other types of replication stress cause fork stalling. In these circumstances, the DNA damage response (DDR) mobilizes repair activities to stabilize the fork, resolve the problem, and complete DNA synthesis (Harper and Elledge 2007; Cimprich and Cortez 2008).

The DDR to replication stress is poorly understood in comparison with the response to double-strand breaks (DSBs). For example, there are extensive modifications to the chromatin surrounding a DSB, including destabilization of nucleosomes, chromatin remodeling, and histone post-translational modifications (Morrison and Shen 2009; van Attikum and Gasser 2009; Rossetto et al. 2010;

Venkitaraman 2010). These changes increase access to the repair machinery and recruit proteins involved in repair and DDR signaling. The extent to which chromatin changes at a stalled fork mimic those at a DSB is unknown.

Replication provides a unique landscape and set of challenges compared with a DSB. The immediate vicinity of the replisome lacks nucleosomes. Also, half of the histones on the nascent DNA are newly synthesized and require changes in post-translational modifications to restore the proper chromatin structure. Finally, several mechanisms exist to recover stalled replication forks, which necessitate the recruitment of multiple enzymatic activities and, perhaps, different chromatin changes.

The difference in our knowledge of the responses at stalled forks compared with DSBs is due primarily to the increased technical challenges of studying replication stress. For example, several investigators have used site-specific DSBs combined with chromatin immunoprecipitation (ChIP) to examine proteins at breaks with high resolution (Rudin and Haber 1988; Rodrigue et al. 2006; Soutoglou et al. 2007; Berkovich et al. 2008). Thus far, site-specific analysis of active and stalled replisomes in mammalian cells has not been achieved. We addressed this technical limitation by developing the iPOND (isolation of proteins on nascent DNA) methodology. iPOND

¹Corresponding author.

E-mail david.cortez@vanderbilt.edu.

Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.205321.1>.

permits the isolation and analysis of proteins at active, stalled, and collapsed replication forks. It can also probe the changes that accompany chromatin deposition and maturation following DNA synthesis. We demonstrate the power of iPOND by defining the dynamics of proteins and post-translational modifications in the replisome and on the newly deposited chromatin.

Results

Development of iPOND

Tracking the location of any single replisome in a mammalian cell is not possible, limiting the utility of ChIP-based technologies. To overcome this technical limitation, we used the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) (Salic and Mitchison 2008), which contains an alkyne functional group. Covalent linkage to a biotin-azide using click chemistry (Moses and Moorhouse 2007) facilitates single-step purification of the EdU-labeled nascent DNA and associated proteins at replication forks (Fig. 1A).

To validate this methodology we first asked whether we could detect replisome proteins. We labeled cells with EdU for 10 min then performed iPOND. We detected proliferating cell nuclear antigen (PCNA), chromatin assembly factor 1 (CAF-1), replication protein A (RPA), and two subunits of polymerase ϵ (Fig. 1B). These results indicate that iPOND can purify replisome proteins, including those indirectly bound to DNA such as CAF-1 (Shibahara and Stillman 1999). Furthermore, they indicate that iPOND is a highly sensitive methodology. We are able to detect proteins such as POLE2 and POLE3, which are expected to be at a density of only one or two molecules per fork (Fig. 1B). Thus, unlike immunofluorescence, iPOND does not require high concentrations of proteins within a small nuclear region to track protein localization. Of note, proteins not present at replication forks, such as GAPDH, are not detectable in iPOND captures (data not shown).

In time-course experiments, we detected PCNA and CAF-1 after a 2.5-min pulse of EdU, histones H2B and H3 after 5 min, and the linker histone H1 at 20 min after EdU addition (Fig. 1C). Thus, with short labeling times, we selectively purify proteins at the replication fork, and longer labeling times permit analysis of chromatin assembly. The order of histone deposition supports previous fractionation data indicating that H1 is added 10–20 min after DNA replication to create higher-order chromatin structures (Worcel et al. 1978).

The resolution of this technique depends on the length of the EdU pulse, the rate of DNA synthesis, and the size of the DNA fragments generated after cell lysis. In practice, the first two parameters are the most important, since we consistently obtain DNA fragments of ~150 base pairs (bp) (Supplemental Fig. 1). In mammalian cells, the rate of DNA synthesis varies between 0.75 and 2.5 kb/min (Herrick and Bensimon 2008). Thus, a 2.5-min EdU pulse labels ~2–6 kb, although this is likely a significant overestimation, since EdU must enter the cell and be phosphorylated before incorporation into DNA. Thus, iPOND resolution is on the order of a few thousand base pairs.

Importantly, iPOND can be combined with pulse-chase methods to track how proteins assemble and disassemble from a nascent DNA segment with high spatial and temporal resolution. Increasing chase times monitor DNA-associated proteins at greater and greater distances from the moving fork. In these experiments, histone levels remain constant, indicating that the procedure effectively captures a maturing chromatin segment of constant length (Fig. 1D). However, PCNA and CAF-1 levels purified with the EdU-labeled segment decline with a half-life of considerably <10 min of chase time (Fig. 1D). These data indicate that iPOND isolates chromatin-associated proteins specifically located at the replication fork, and are consistent with rapid unloading of PCNA and CAF-1 once Okazaki fragment DNA synthesis is complete.

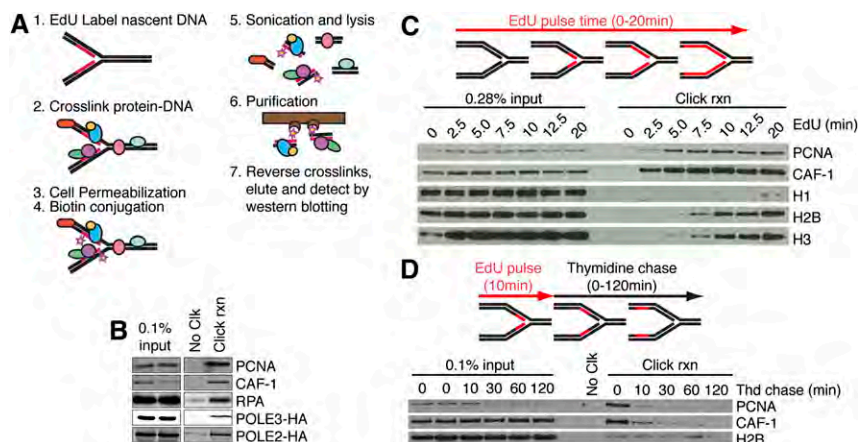


Figure 1. Development of the iPOND technology. (A) iPOND begins by adding EdU to cultured cells. The cells are then treated with formaldehyde to cross-link protein-DNA complexes, washed, and permeabilized with detergent. Copper catalyzes the cycloaddition of biotin-azide to the EdU-labeled DNA. The cells are then lysed in denaturing conditions with sonication. The biotin-labeled DNA-protein complexes are purified using streptavidin-coated beads, cross-links are reversed, and the eluted proteins are analyzed by immunoblotting or other methods like mass spectrometry. (B) Cells were incubated with EdU for 10 min prior to performing iPOND. Cells expressing POLE2-HA or POLE3-HA were used to detect these proteins with the

HA antibody. (C) Cells were incubated in EdU-containing medium for increasing times prior to performing the iPOND protocol. (D) Cells were incubated with EdU for 10 min. The EdU-containing medium was removed and cells were washed once before incubating for increasing times in medium containing 10 μ M thymidine prior to performing iPOND. In all experiments, the No Clk control is the input sample in the first lane processed with no biotin-azide.

Sirbu et al.

Analysis of chromatin maturation using iPOND

Maturation of the new chromatin requires addition and removal of histone post-translational modifications. Newly synthesized histone H4 is acetylated on two lysines (5 and 12), and these evolutionarily conserved marks are removed after deposition (Sobel et al. 1995; Taddei et al. 1999). Our time course experiments indicate that acetylated H4K5 (H4K5ac) is removed rapidly and H4K12ac deacetylation is slightly delayed (Fig. 2A,B). The delay in K12 deacetylation could be due to the activity of chromatin-associated histone acetyltransferases (HATs) that promote the acetylation of this site in some chromatin domains. Indeed, in the presence of the nonselective HAT inhibitor anacardic acid, the rate of H4K12 deacetylation becomes identical to H4K5, with a half-life of <20 min (Fig. 2C,D).

In principle, chromatin maturation—as measured by H4K5,K12 deacetylation—could be coupled to fork progression. To test this possibility, we used high concentrations of hydroxyurea (HU) to stall active replisomes and stop DNA synthesis. HU addition stalls the fork effectively in these cells, since the amount of histone capture does not increase appreciably during the HU treatment (Fig. 2E). Deacetylation of newly deposited H4 proceeds at the same rate regardless of whether DNA synthesis is inhibited. Thus, chromatin maturation can be uncoupled from replisome movement.

The histone deacetylase (HDAC) in human cells that catalyzes the deacetylation of H4K5 and K12 is unknown. HDAC1 and HDAC2 associate with CAF-1 (Ahmad et al. 1999), and HDAC3 is required—perhaps in late S phase or G2—to remove H4K5ac (Bhaskara et al. 2010). Indeed, in pulse-chase experiments, we found an enrichment of

HDAC1, HDAC2, and HDAC3 near the fork (Fig. 2A), and the selective class I HDAC inhibitor FK228 (Furumai et al. 2002) prevented deacetylation of H4 (Fig. 2F), suggesting that all three of these HDACs are involved.

DDR response at stalled replication forks

HU treatment causes DDR activation to stabilize the stalled fork and induce a cell cycle checkpoint. Previous studies suggest that HU-stalled forks remain stable and competent to resume DNA synthesis for several hours; however, eventually, the stalled fork collapses and DSBs are formed (Petermann et al. 2010). To further examine this process, we monitored recruitment and modification of proteins at stalled forks. The amounts of PCNA and CAF-1 that are captured at the stalled fork decrease initially after adding HU to the medium, and then reach a steady state level of between 20% and 30% of that found at an elongating fork (Fig. 3A). This PCNA pattern is likely due to unloading of PCNA from the completed Okazaki fragments. We detected RPA associated with the fork both before and after HU addition (Fig. 3A). The amount of RPA detected remained constant even though RPA accumulates at stalled forks (Cimprich and Cortez 2008). This discrepancy is explained because RPA binds only to the single-stranded, template strand of DNA, which lacks incorporated EdU. Therefore, iPOND detects only the RPA immediately adjacent to the newly synthesized dsDNA (Supplemental Fig. 2).

In these experiments, we noticed that at 120 and 240 min after addition of HU, the electrophoretic mobility of RPA decreased, consistent with phosphorylation (Fig. 3A). RPA S33 phosphorylation could be detected within 10 min of HU addition, and S4/S8 phosphorylation appeared at 2 h

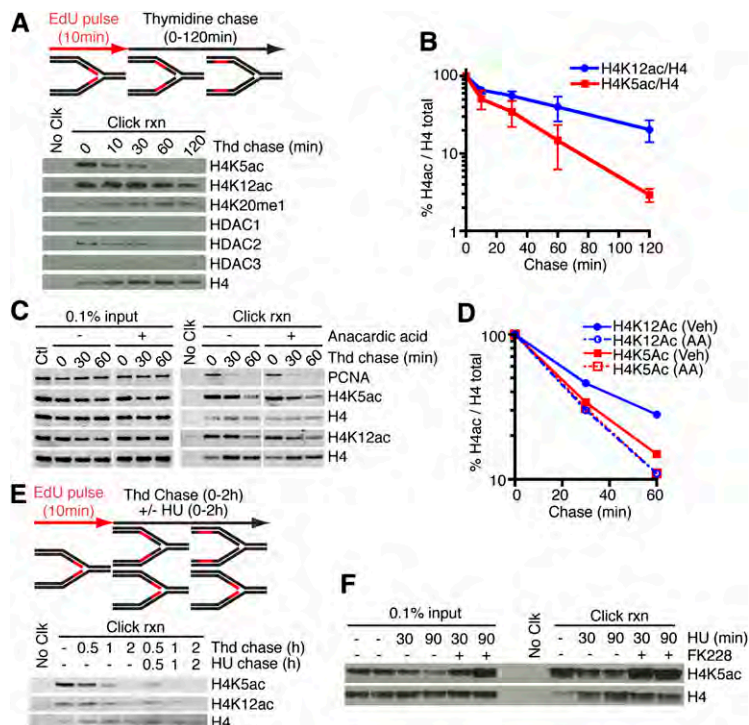


Figure 2. HDACs are enriched at replication forks and deacetylate newly deposited histone H4 regardless of fork movement. (A–E). Cells were labeled with EdU for 10 min followed by a chase into thymidine-containing medium for the indicated times prior to performing iPOND. (B) Quantitation of H4 acetylation levels compared with total H4 in the click reaction samples from three independent experiments. Error bars in all figures are standard deviations. (C,D) Anacardic acid (30 μ M) was added to the indicated samples. (E) HU (3 mM) was added to the indicated samples. (F) Cells labeled with EdU were chased into 3 mM HU medium with or without 100 nM FK228 prior to performing iPOND.

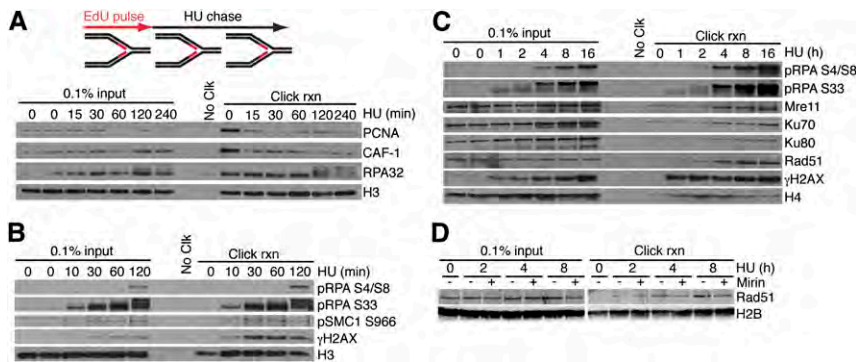


Figure 3. iPOND monitors post-translational modifications and recruitment of DDR proteins to stalled and collapsed replication forks. (A–D) Cells were labeled with EdU for 15 min (A) or 10 min (B–D), followed by a chase into HU for the indicated times prior to performing iPOND. (D) HU-treated cells were additionally coincubated with or without the Mre11 inhibitor mirin (100 μ M) as indicated.

(Fig. 3B). DNA-PK catalyzes S4/S8 phosphorylation and ATR catalyzes S33 phosphorylation (Anantha et al. 2007), suggesting that ATR phosphorylates RPA immediately after fork stalling, and DNA-PK phosphorylates RPA only at persistently stalled forks.

H2AX phosphorylation (γ H2AX) is often considered a marker for DSBs (Dickey et al. 2009). However, we observed γ H2AX at stalled replication forks at even the earliest time points (10 min) after HU addition (Fig. 3B), well before evidence of DSB formation (Petermann et al. 2010). These data prompted us to examine the timing of recruitment of DSB repair proteins. MRE11, KU70, and KU80 exhibited a recruitment profile in which low amounts were observable before the addition of HU, and remained unchanged for 2 h after HU addition (Fig. 3C). However, by 4 h in HU, we detected a significant increase in all of these proteins near the stalled fork (Fig. 3C). RAD51 was first detectable after HU addition, but its levels also increased significantly by 4 h, suggesting that DSBs may form between 2 and 4 h after the fork is stalled. KU70 and KU80 may bind to some of the single-ended breaks, and RAD51 may bind to others.

At DSBs, MRE11-dependent end resection is required to load RAD51 (Mimitou and Symington 2009). At collapsed forks, RAD51 may function to promote recombination-based methods to re-establish the replication fork (Errico and Costanzo 2010). To test whether the loading of RAD51 at stalled forks also requires MRE11, we treated cells with the MRE11 nuclease inhibitor mirin (Dupre et al. 2008). Although the early recruitment of RAD51 occurred independently of MRE11, the late accumulation required MRE11 activity (Fig. 3D), suggesting that end resection promotes this loading. The timing of MRE11 recruitment also correlated with a large increase in RPA S4/S8 phosphorylation (Fig. 3C), which was previously linked to end resection at camptothecin-damaged forks (Sartori et al. 2007).

γ H2AX spreading from stalled forks before and after fork collapse

We noticed that the rapid phosphorylation of H2AX near the fork saturates within 30 min; however, global levels continue to increase (Fig. 3B, cf. the click rxn lanes and the input lanes). Therefore, we hypothesized that the

global increase stems from γ H2AX spreading from the stalled fork, as is observed near DSBs (Berkovich et al. 2007; Savic et al. 2009). To test this hypothesis, we first labeled cells with EdU, then chased with thymidine for various lengths of time to extend the distance between the EdU-labeled fragment and the fork, and finally added HU to stall the fork. We again observed maximum γ H2AX at the fork 30 min after HU addition; however, the chromatin region distant from the fork contained low but detectable levels of γ H2AX that increased when examined at 60 min after HU addition (Fig. 4A, cf. lanes 4–6 and 7–9). A more detailed analysis revealed that the density of γ H2AX gradually declined as a function of distance from the stalled fork (Fig. 4B,C). Compared with the saturated density at the fork, the γ H2AX density decreased approximately twofold for every 15 min of thymidine chase time when cells were treated with HU for 1 h. By 2 h, we observed increased γ H2AX density in all chromatin segments analyzed, suggesting that γ H2AX spreading contributes significantly to the global change in γ H2AX levels.

To examine the chromatin at a single location distant from the fork, we repeated this experiment holding the thymidine chase time constant at 30 min, and treated with HU for varying times. We observed a steady increase in γ H2AX at this distance from the fork (Fig. 4D). Importantly, these results indicate considerable spreading of the γ H2AX signal even shortly after fork stalling. Assuming a conservative rate of fork elongation of 1 kb/min, these data imply that, within 1 h of fork stalling, γ H2AX spreads to include a large domain containing tens of thousands of base pairs of DNA.

To identify the kinases that phosphorylate H2AX adjacent to the stalled fork and that promote spreading, we used small molecule kinase inhibitors. The selective DNA-PK and ATM inhibitors NU7441 (Leahy et al. 2004) and KU55933 (Hickson et al. 2004) had minimal effects on the spreading or total levels of γ H2AX induced by a short (30- to 60-min) HU treatment (Fig. 5A; Supplemental Fig. 3A). However, these inhibitors did significantly reduce γ H2AX levels at all chromosomal positions relative to the fork in cells treated with HU for 4 h (Fig. 5B,C; Supplemental Fig. 3B). These results indicate that DNA-PK/ATM contributes to maintenance and spreading of γ H2AX at persistently stalled forks. In contrast,

Sirbu et al.

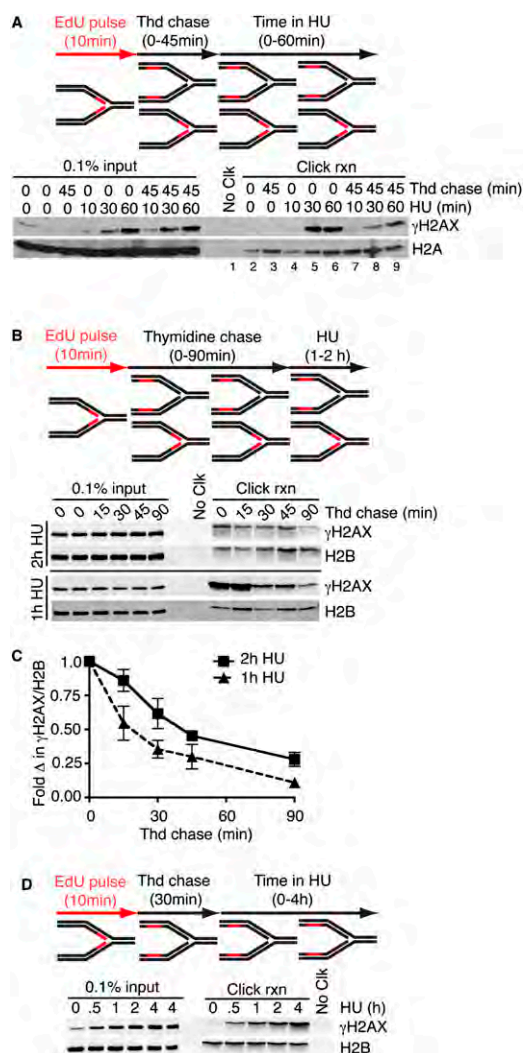


Figure 4. γ H2AX spreads from a stalled replication fork. (A–D) Cells labeled with EdU for 10 min were chased into thymidine-containing medium prior to addition of HU, then processed using iPOND. The length of the thymidine and HU treatments is indicated. Quantitation of the click reaction samples in C at the 2-h HU-treated samples is from three independent experiments, and at the 1-h HU-treated samples is from two independent experiments.

treatment with caffeine, which preferentially inhibits ATR (Sarkaria et al. 1999), significantly reduced γ H2AX formation and spreading shortly after the fork is stalled (Fig. 5D). These results are consistent with a model in which ATR phosphorylates H2AX at a stalled fork and promotes initial spreading. At later time points, when DSBs likely form at the fork, ATM and DNA-PKcs maintain and further propagate the H2AX phosphorylation (Supplemental Fig. 4).

Discussion

Previous studies of the replisome and DDR responses at stalled forks relied largely on immunofluorescent imag-

ing to track protein localization. While useful, immunofluorescence has the significant disadvantages of low resolution and low sensitivity. For example, proteins that exist at only single-copy levels at replication forks cannot be tracked with immunofluorescent imaging. In contrast, iPOND technology has dramatically improved sensitivity,

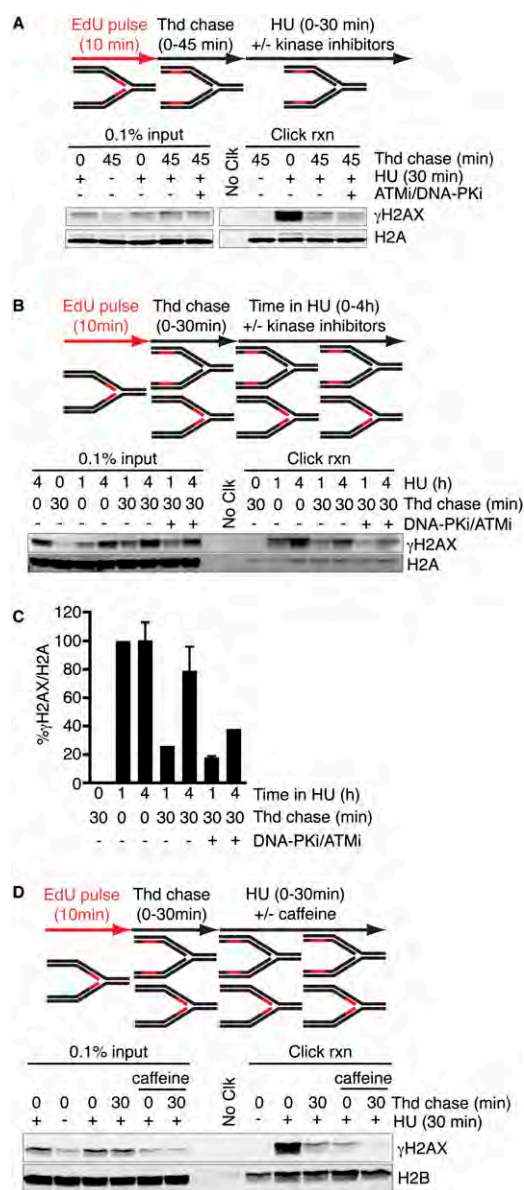


Figure 5. Checkpoint kinases propagate H2AX phosphorylation from stalled replication forks. (A–C) Cells labeled with EdU for 10 min were chased into thymidine, followed by treatment with HU. The length of thymidine and HU treatments are indicated. DNA-PK (KU7441, 1 μ M) and ATM (KU5593, 10 μ M) inhibitors were added at the same time as HU in the indicated samples. (C) Quantitation of the click reaction samples is the average from two independent experiments and is normalized to the 1-h HU treatment. (D) Cells labeled with EdU for 10 min were chased into thymidine for either 0 or 30 min, followed by a 30-min treatment with HU. Caffeine (10 mM) was added at the same time as HU in the indicated samples.

allowing us to detect even proteins such as polymerases. Furthermore, combining iPOND with pulse-chase methods provides high spatial and temporal resolution of protein dynamics. Finally, iPOND also facilitates analysis of post-translational modifications, which is often impossible with immunofluorescent imaging due to poor antibody quality or specificity.

Recently, the Helleday group (Petermann and Helleday 2010) isolated CldU-labeled DNA using an antibody to show that Rad51 is bound to recently synthesized DNA. However, they used a 40-min labeling time, so it is unclear whether this method is sufficiently sensitive or specific to produce high spatial and temporal resolution like iPOND. Also, unlike CldU-IP, iPOND does not require ssDNA to permit antibody access to an antibody epitope, and the biotin–streptavidin purification procedure is compatible with highly stringent conditions (1% SDS and 1 M NaCl), thereby improving specificity.

iPOND is an ensemble methodology. Thus, it provides an average picture of events in different cells at stalled forks throughout the genome. iPOND can be combined with cell synchronization to examine replication and chromatin maturation in early and late replicating genomic regions. In principle, iPOND should be applicable to any process involving DNA synthesis, such as analysis of DNA excision repair.

A disadvantage of iPOND over ChIP methods is the lack of a PCR amplification step. Thus, much larger amounts of input material are necessary to achieve sufficient protein for detection. Fortunately, the covalent coupling of EdU and biotin during the click reaction permits a single-step, highly efficient purification in stringent buffer, salt, and detergent conditions. A significant advantage of iPOND compared with ChIP is its compatibility with unbiased screening approaches. We anticipate coupling iPOND to mass spectrometry to identify all proteins at active and damaged replisomes. Furthermore, mass spectrometry analysis of iPOND-captured histones will facilitate studies of chromatin assembly and maturation.

Chromatin assembly is thought to occur by a stepwise deposition of the core histones, followed by linker histones and changes in post-translational modifications (Probst et al. 2009). Our data confirm this assembly process *in vivo* in cultured mammalian cells. Furthermore, we found that at least some chromatin maturation processes, such as the removal of acetylation on H4K5 and H4K12, proceed even when decoupled from replisome movement. HDAC1, HDAC2, and HDAC3 are enriched on newly synthesized DNA, and an inhibitor that targets all three of these enzymes prevents H4K5ac and H4K12ac deacetylation. Intriguingly, deacetylation of H4K5ac and H4K12ac occurred at the same rate, but acetyltransferases rapidly reacylated H4K12, suggesting a specific need for this modification in some chromatin domains.

In the yeast *Saccharomyces cerevisiae*, H3K56 acetylation is also associated with newly deposited histones during DNA replication, and promotes survival in response to replication stress (Masumoto et al. 2005). We were unable to detect this acetylation mark on newly deposited histones or after HU treatment (data not shown).

This observation is consistent with other human cell studies that found low levels of this post-translational modification in total chromatin that further decreased in response to DNA damage (Tjeertes et al. 2009).

Prominent changes in response to replication stress include protein phosphorylation. Importantly, our data indicate that H2AX phosphorylation spreads to a large chromatin domain early in the response to fork stalling. This early phosphorylation is catalyzed by ATR and is unlikely to be due to the processing of the fork into a DSB intermediate. Our data are consistent with previous analyses implicating both ATR-dependent (Ward and Chen 2001) and ATR-independent (Brown and Baltimore 2003; Gilad et al. 2010) H2AX phosphorylating activities in response to fork arrest. Most models of ATR function suggest that it is active only when bound to the ssDNA at the stalled fork through an ATRIP–RPA interaction (Cimprich and Cortez 2008), but our data indicate that ATR helps spread the γ H2AX signal. One possibility is that the early spreading of γ H2AX is due to looping of the newly synthesized chromatin that brings it into proximity of ATR. Alternatively, ATR may have a method of spreading its signal beyond the immediate ssDNA vicinity, similar to the ability of active ATM to spread along the dsDNA away from the DSB end (You et al. 2007). MDC1 may be involved in such a process (Ichijima et al. 2011; Wang et al. 2011).

Persistent stalling of the fork for longer than 1–2 h causes a switch in the DDR. RPA is hyperphosphorylated on DNA-PK-dependent phosphorylation sites, ATM/DNA-PK catalyzes further γ H2AX spreading, and DSB repair proteins like MRE11, KU70/80, and RAD51 accumulate. RAD51 assembly at these persistently stalled forks depends on MRE11 activity, suggesting a requirement for end resection. The end resection may be on the template DNA strand, since we continued to capture EdU-labeled DNA and associated proteins. Resecting the leading strand template would yield a 3' overhang of newly synthesized DNA, which could be used in recombination-based methods of fork repair and restart (Petermann and Helleday 2010).

Overall, these data provide the first high-resolution, time-dependent analyses of protein dynamics at active, stalled, and collapsed replication forks in mammalian cells. Furthermore, they validate iPOND as a powerful method to study DDRs, chromatin deposition, and chromatin maturation during DNA replication.

Materials and methods

Cell culture

HEK293T cells were cultured in DMEM supplemented with 7.5% FBS. Stable cell lines expressing POLE2-HA and POLE3-HA were generated by retroviral infection and selection in puromycin-containing medium.

Plasmid constructs

POLE2-HA and POLE3-HA retroviral vectors were generated by gateway cloning. pENTR POLE2 and pENTR POLE3 were

Sirbu et al.

recombined with pLPCX-GW-HA3X (pDC1127) to generate a C-terminal HA-tagged POLE2 and POLE3 retroviral vectors. pDC1127 was created by subcloning a 3XHA epitope into pLPCX between the NotI and ClaI restriction sites, then subcloning the gateway cassette containing attR1, *ccdB* gene, and attR2 as an EcoRV fragment between EcoRI and NotI sites.

iPOND

EdU-labeled sample preparation HEK 293T cells ($\sim 1.5 \times 10^8$ cells per sample) were incubated with 10–12 μ M EdU (Vanderbilt Synthesis Core). For pulse-chase experiments with thymidine (Sigma), EdU-labeled cells were washed once with temperature- and pH-equilibrated medium containing 10 μ M thymidine to remove the EdU, then chased into 10 μ M thymidine. Other chemicals were added to the cell cultures at the following concentrations: HU (3 mM; Sigma), HAT inhibitor anacardic acid (30 μ M; Enzo), HDAC inhibitor FK228 (100 nM; kindly provided by Dineo Khabele), Mre11 inhibitor Mirin (100 μ M; Sigma), ATM inhibitor (KU55933, 10 μ M; AstraZeneca), DNA-PK inhibitor (KU57788, 1 μ M; AstraZeneca), and caffeine (10 mM; ICN Biomedicals). DMSO was used as a vehicle control where appropriate.

After labeling, cells were cross-linked in 1% formaldehyde/PBS for 20 min at room temperature, quenched using 0.125 M glycine, and washed three times in PBS. Collected cell pellets were frozen at -80°C , then resuspended in 0.25% Triton-X/PBS to permeabilize. Pellets were washed once with 0.5% BSA/PBS and once with PBS prior to the click reaction.

Click reaction Cells were incubated in click reaction buffer for 1–2 h at a concentration of 2×10^7 cells per milliliter of click reaction buffer. The click reaction buffer contains Invitrogen's Click-iT cell reaction buffer and cell buffer additive (C10269), 2 mM copper (II) sulfate (CuSO_4), and 1 μ M photocleavable biotin-azide (Kim et al. 2009) (kindly provided by Ned Porter). DMSO was added instead of biotin-azide to the negative control samples (no clk in all figures). Cell pellets were washed once with 0.5% BSA/PBS and once with PBS.

Cell lysis Cells were then resuspended in lysis buffer containing 1% SDS, 50 mM Tris (pH 8.0), 1 μ g/mL leupeptin, and 1 μ g/mL aprotinin. Samples were sonicated (Micro-tip, Misonix 4000 or Fisher Scientific Sonic Dismembrator model 500) using the following settings: 13–16 W, 20-sec constant pulse, and 40- to 59-sec pause for a total of 4–5 min. Samples were centrifuged at 13,200 rpm for 10 min, filtered through a 90- μ m nylon mesh, and diluted 1:1 (v/v) with PBS containing 1 μ g/mL leupeptin and 1 μ g/mL aprotinin prior to purification.

Purification Streptavidin–agarose beads (Novagen) were washed 1:1 (v/v) twice in lysis buffer and once in PBS. Washed beads were incubated with the samples for 14–20 h at 4°C in the dark. The beads were washed once with lysis buffer, once with 1 M NaCl, and then twice with lysis buffer. Captured proteins were eluted and cross-links were reversed in SDS sample buffer by incubating for 25 min at 95°C . Proteins were resolved on SDS-PAGE and detected by immunoblotting. In most cases, quantitative immunoblotting was performed using the Odyssey infrared imaging system.

Antibodies

Antibodies used were as follows: PCNA (Santa Cruz Biotechnology); CAF-1 p60, RPA32, pRPA32 S4/S8, pRPA32 S33, and pSMC1 S966 (Bethyl Laboratories); FK2 (Calbiochem); RAD51,

H2B, H2A, H3, H4, H4K5Ac, KU70, KU80, HDAC1, HDAC2, and HDAC3 (Abcam); γ H2AX, H1 (Millipore); MRE11 (Genetex); H4K12Ac and H4K20me1 (Active Motif); and anti-HA (Covance).

Determination of DNA fragment size

To determine DNA fragment size, 5 μ L of pre- and post-sonication samples were incubated at 65°C to reverse the DNA–protein cross-links, then incubated with RNaseA and proteinase K. DNA samples were resolved on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. DNA fragment sizes ranged between 100–300 bp. It should be noted that we determined that the CuSO_4 in the click reaction catalyzes cleavage of the phosphodiester bond and assists in generating the small fragment size.

Acknowledgments

We thank Drs. Ned Porter, Keri Tallman, Dineo Khabele, Janel McLean, Kathy Gould, Mahesh Chandrasekharan, Simona Codreanu, Daniel Liebler, and Larry Marnett for supplying reagents and advice. This work was supported by NIH grant R01CA136933 to D.C. Additional support was provided by the Vanderbilt-Ingram Cancer Center (CA06485) and the Ingram Charitable Fund. B.M.S. is supported by a Department of Defense Breast Cancer Research Program predoctoral fellowship (W81XWH-10-1-0226). F.B.C. is supported by the Molecular Toxicology training grant (T32 CA09582).

References

- Ahmad A, Takami Y, Nakayama T. 1999. WD repeats of the p48 subunit of chicken chromatin assembly factor-1 required for in vitro interaction with chicken histone deacetylase-2. *J Biol Chem* **274**: 16646–16653.
- Anantha RW, Vassin VM, Borowiec JA. 2007. Sequential and synergistic modification of human RPA stimulates chromosomal DNA repair. *J Biol Chem* **282**: 35910–35923.
- Berkovich E, Monnat RJ Jr, Kastan MB. 2007. Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. *Nat Cell Biol* **9**: 683–690.
- Berkovich E, Monnat RJ Jr, Kastan MB. 2008. Assessment of protein dynamics and DNA repair following generation of DNA double-strand breaks at defined genomic sites. *Nat Protoc* **3**: 915–922.
- Bhaskara S, Knutson SK, Jiang G, Chandrasekharan MB, Wilson AJ, Zheng S, Yenamandra A, Locke K, Yuan JL, Bonine-Summers AR, et al. 2010. Hdac3 is essential for the maintenance of chromatin structure and genome stability. *Cancer Cell* **18**: 436–447.
- Brown EJ, Baltimore D. 2003. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev* **17**: 615–628.
- Cimprich KA, Cortez D. 2008. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* **9**: 616–627.
- Dickey JS, Redon CE, Nakamura AJ, Baird BJ, Sedelnikova OA, Bonner WM. 2009. H2AX: functional roles and potential applications. *Chromosoma* **118**: 683–692.
- Dupre A, Boyer-Chatenet L, Sattler RM, Modi AP, Lee JH, Nicolette ML, Kopelovich L, Jasim M, Baer R, Paull TT, et al. 2008. A forward chemical genetic screen reveals an inhibitor of the Mre11-Rad50-Nbs1 complex. *Nat Chem Biol* **4**: 119–125.
- Errico A, Costanzo V. 2010. Differences in the DNA replication of unicellular eukaryotes and metazoans: known unknowns. *EMBO Rep* **11**: 270–278.

- Furumai R, Matsuyama A, Kobashi N, Lee KH, Nishiyama M, Nakajima H, Tanaka A, Komatsu Y, Nishino N, Yoshida M, et al. 2002. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. *Cancer Res* **62**: 4916–4921.
- Gilad O, Nabet BY, Ragland RL, Schoppy DW, Smith KD, Durham AC, Brown EJ. 2010. Combining ATR suppression with oncogenic Ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner. *Cancer Res* **70**: 9693–9702.
- Harper JW, Elledge SJ. 2007. The DNA damage response: ten years after. *Mol Cell* **28**: 739–745.
- Herrick J, Bensimon A. 2008. Global regulation of genome duplication in eukaryotes: an overview from the epifluorescence microscope. *Chromosoma* **117**: 243–260.
- Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ, Smith GC. 2004. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res* **64**: 9152–9159.
- Ichijima Y, Ichijima M, Lou Z, Nussenzweig A, Camerini-Otero RD, Chen J, Andreassen PR, Namekawa SH. 2011. MDC1 directs chromosome-wide silencing of the sex chromosomes in male germ cells. *Genes Dev* **25**: 959–971.
- Kim HY, Tallman KA, Liebler DC, Porter NA. 2009. An azido-biotin reagent for use in the isolation of protein adducts of lipid-derived electrophiles by streptavidin catch and photo-release. *Mol Cell Proteomics* **8**: 2080–2089.
- Leahy JJ, Golding BT, Griffin RJ, Hardcastle IR, Richardson C, Rigoreau L, Smith GC. 2004. Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. *Bioorg Med Chem Lett* **14**: 6083–6087.
- Masumoto H, Hawke D, Kobayashi R, Verreault A. 2005. A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature* **436**: 294–298.
- Mimitou EP, Symington LS. 2009. DNA end resection: many nucleases make light work. *DNA Repair (Amst)* **8**: 983–995.
- Morrison AJ, Shen X. 2009. Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. *Nat Rev Mol Cell Biol* **10**: 373–384.
- Moses JE, Moorhouse AD. 2007. The growing applications of click chemistry. *Chem Soc Rev* **36**: 1249–1262.
- Petermann E, Helleday T. 2010. Pathways of mammalian replication fork restart. *Nat Rev Mol Cell Biol* **11**: 683–687.
- Petermann E, Orta ML, Issaeva N, Schultz N, Helleday T. 2010. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol Cell* **37**: 492–502.
- Probst AV, Dunleavy E, Almouzni G. 2009. Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* **10**: 192–206.
- Rodrigue A, Lafrance M, Gauthier MC, McDonald D, Hendzel M, West SC, Jasin M, Masson JY. 2006. Interplay between human DNA repair proteins at a unique double-strand break in vivo. *EMBO J* **25**: 222–231.
- Rossetto D, Truman AW, Kron SJ, Cote J. 2010. Epigenetic modifications in double-strand break DNA damage signaling and repair. *Clin Cancer Res* **16**: 4543–4552.
- Rudin N, Haber JE. 1988. Efficient repair of HO-induced chromosomal breaks in *Saccharomyces cerevisiae* by recombination between flanking homologous sequences. *Mol Cell Biol* **8**: 3918–3928.
- Salic A, Mitchison TJ. 2008. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc Natl Acad Sci* **105**: 2415–2420.
- Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT. 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res* **59**: 4375–4382.
- Sartori AA, Lukas C, Coates J, Mistrik M, Fu S, Bartek J, Baer R, Lukas J, Jackson SP. 2007. Human CtIP promotes DNA end resection. *Nature* **450**: 509–514.
- Savic V, Yin B, Maas NL, Bredemeyer AL, Carpenter AC, Helmink BA, Yang-Iott KS, Sleckman BP, Bassing CH. 2009. Formation of dynamic γ -H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent upon H2AX densities in chromatin. *Mol Cell* **34**: 298–310.
- Shibahara K, Stillman B. 1999. Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96**: 575–585.
- Sobel RE, Cook RG, Perry CA, Annunziato AT, Allis CD. 1995. Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *Proc Natl Acad Sci* **92**: 1237–1241.
- Soutoglou E, Dorn JF, Sengupta K, Jasin M, Nussenzweig A, Ried T, Danuser G, Misteli T. 2007. Positional stability of single double-strand breaks in mammalian cells. *Nat Cell Biol* **9**: 675–682.
- Taddei A, Roche D, Sibarita JB, Turner BM, Almouzni G. 1999. Duplication and maintenance of heterochromatin domains. *J Cell Biol* **147**: 1153–1166.
- Tjeertes JV, Miller KM, Jackson SP. 2009. Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. *EMBO J* **28**: 1878–1889.
- van Attikum H, Gasser SM. 2009. Crosstalk between histone modifications during the DNA damage response. *Trends Cell Biol* **19**: 207–217.
- Venkitaraman AR. 2010. Modifying chromatin architecture during the response to DNA breakage. *Crit Rev Biochem Mol Biol* **45**: 2–13.
- Wang J, Gong Z, Chen J. 2011. MDC1 collaborates with TopBP1 in DNA replication checkpoint control. *J Cell Biol* **193**: 267–273.
- Ward IM, Chen J. 2001. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem* **276**: 47759–47762.
- Worcel A, Han S, Wong ML. 1978. Assembly of newly replicated chromatin. *Cell* **15**: 969–977.
- You Z, Bailis JM, Johnson SA, Dilworth SM, Hunter T. 2007. Rapid activation of ATM on DNA flanking double-strand breaks. *Nat Cell Biol* **9**: 1311–1318.